Receptors of Vascular Endothelial Growth Factor/Vascular Permeability Factor (VEGF/VPF) in Fetal and Adult Human Kidney: Localization and [^{125}I]VEGF Binding Sites

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Abstract. Vascular endothelial growth factor (VEGF) has an important function in renal vascular ontogenesis and is constitutively expressed in podocytes of the adult kidney. The ability of VEGF to be chemotactic for monocytes and to increase the activity of collagenase and plasminogen activator may have implications for renal development and renal disease. In humans, the cellular actions of VEGF depend on binding to two specific receptors: Flt-1 and KDR. The aims of this study were: (*1*) to localize VEGF receptor proteins in human renal ontogenesis; (*2*) to quantify VEGF binding in human fetal and adult kidney; and (*3*) to dissect the binding into its two known components: the KDR and Flt-1 receptors. The latter aim was achieved by competitive binding of VEGF and placenta growth factor-2, which only binds to Flt-1. Quantification of ^{125}I-VEGF binding sites was performed by autoradiography and computerized densitometry. By double-label immunohistochemistry, VEGF receptor proteins were localized solely to endothelial cells of preglomerular vessels, glomeruli, and postglomerular vessels. In developing glomeruli, VEGF receptor protein appeared as soon as endothelial cells were positive for von Willebrand factor. Specific ^{125}I-VEGF binding could be localized to renal arteries and veins, glomeruli, and the tubulointerstitial capillary network in different developmental stages. Affinity (K_d) of adult (aK) and fetal (fK) kidneys was: K_d: glomeruli 38.6 ± 11.2 (aK, n = 5), 36.3 ± 7.1 (fK, n = 5); cortical tubulointerstitium 19.4 ± 2.6 (aK, n = 5), 11.6 ± 7.0 (fK, n = 5) pmol. Placenta growth factor-2 displaced VEGF binding in all renal structures by approximately 60%. VEGF receptor proteins thus were found only in renal endothelial cells. A coexpression of both VEGF binding sites could be shown, with Flt-1 demonstrating the most abundant VEGF receptor binding sites in the kidney. These studies support the hypothesis of a function for VEGF in adult kidney that is independent of angiogenesis. (J Am Soc Nephrol 9: 1032–1044, 1998)

The kidney possesses a complex vascular system with distinct functional and morphologic characteristics. The endothelial cells of glomerular and peritubular capillaries are fenestrated (1). Glomerular capillaries are exposed to a high transcapillary pressure (2). The development and integrity of this renal blood vessel system seems to be regulated by growth factors/cytokines and their receptors (3).

Vascular endothelial growth factor (VEGF) is a potent mitogen for endothelial cells (4,5). It is also known as vascular permeability factor (VPF) because of its ability to increase microvascular permeability (6). *In situ* studies and phenotypes of transgenic mice lacking a functional gene for VEGF point to an important function for VEGF in vascular ontogenesis and kidney development (3,7,8).

VEGF is a dimeric protein with a molecular weight of 34 to 46 kD. Four molecular isoforms of VEGF ($VEGF_{121}$, $_{165}$, $_{189}$, and $_{206}$ [indicating number of amino acids]) have been detected (9). The two smaller VEGF isoforms are secreted and are readily measurable in medium of transfected cells. Although the two larger forms also display a typical leader sequence preceding the NH_2 terminus, these two isoforms are cell-associated. This is probably because of a 24-amino acid region homologous to exon 6 of platelet-derived growth factor (PDGF)-A chain enriched with basic residues that apparently increase binding to glycosaminoglycans on the plasma membrane (10).

VEGF mRNA and protein have been demonstrated in meso-

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and metanephros in glomerular visceral epithelia and in collecting duct epithelia by *in situ* hybridization and immunohistochemistry (11,12).

Recombinant VEGF protein given to rabbit embryonic kidney cortex leads to development of capillaries in organotypic culture conditions (13); in contrast, *in vivo* inhibition of VEGF activity by antibody leads to abnormal glomeruli lacking capillary tufts (14).

The ability of VEGF to be chemotactic for monocytes and to increase the activity of collagenase and plasminogen activator may also have implications for normal kidney development and the pathogenesis of renal disease (15–18).

VEGF binds selectively and with high affinity to two plasma membrane receptors called Flt-1 (fms-like tyrosine kinase-1 = VEGF receptor [R] 1) and flk-1 (fetal liver kinase = VEGF receptor [R] 2) in mouse or KDR (kinase insert domain receptor) in humans. These receptors are type III tyrosine kinase receptors; their extracellular domains are characterized by seven immunoglobulin-like repeats (19,20).

Both receptors have intracellular domains with tyrosine kinase activity. Results on knockout mice suggest that KDR/flk-1 is important for vasculogenesis, whereas Flt-1 is essential to mediate endothelial cell-to-cell and cell-to-matrix interactions (21,22). Because KDR and Flt-1 also may be expressed on nonendothelial cells such as monocytes, beta cells of pancreatic islet cells, mesangial cells, and uterine smooth muscle cells, VEGF may exert other effects in addition to modulation of endothelial cell proliferation and integrity (23–25). *In situ* studies for VEGF receptor mRNA in human kidney suggest that VEGF receptors are exclusively found on endothelial cells.

For the human kidney, VEGF receptor proteins have not been localized, and their activity has not been characterized. Knowledge of these aspects of renal VEGF receptors is part of the basic data for an analysis of VEGF function in human kidney. Cultured endothelial cells from different tissues and sources have been used to generally define the combined binding characteristics of these receptors (26). As cells in culture can change their morphologic and functional features compared with the *in vivo* situation, receptor binding studies *in vitro* cannot always be transferred to the *in vivo* situation.

VEGF/VPF binding studies have been performed in the adult rat, using human recombinant $VEGF_{165}$ obtained from conditioned medium of transfected Chinese hamster ovary cells (27). With regard to receptor binding for vasoactive peptides, we have shown that there are important differences between rodents, and highly developed mammals and humans (28,29).

Therefore, the aims of our study were: (*1*) to localize VEGF receptor proteins by immunohistochemistry, using specific polyclonal and monoclonal antibodies; (2) to quantify VEGF binding in human kidney during ontogeny and in the adult by an *in situ* quantitative radiographic method; and (*3*) to dissect the binding into two components, one due to KDR and one due to Flt-1. The third aim was achieved by competitive binding of VEGF and placenta growth factor (PLGF)-2. PLGF-2, like VEGF, is an endothelial cell growth factor with a 53% identity in amino acid sequence to VEGF (30,31). VEGF glycosylated homodimers bind with high affinity to Flt-1 (VEGF-R1) and KDR (VEGF-R2); in contrast, PLGF binds to the extracellular domain of Flt-1, but not to KDR, with high affinity (32). These receptor-binding features of PLGF make it possible to quantify the binding sites of the VEGF receptors Flt-1 and KDR separately.

Materials and Methods

Preparation of Antibodies against the Soluble KDR and Flt-1 Receptors

Generation of Soluble VEGF Receptors. KDR was cloned from cDNA from a human placenta cDNA library. A 2.3-kb *Bgl*II fragment coding for the first seven IgG-like extracellular domains was transferred into the vector pBacPAK9 (Clontech, Heidelberg, Germany) for baculovirus expression. After transfection into Sf158 insect cells, clones were obtained by screening for the presence of KDR message. These clones were used for virus amplification and overexpression of soluble KDR (sKDR). Insect cell supernatants were purified (33). The identity of the purified protein with an apparent molecular mass of 110 kD with sKDR was shown by N-terminal sequence analysis, using Edman degradation chemistry on an automatic gas sequencer (Applied Biosystems, Weiterstadt, Germany). The human soluble Flt-1 protein (domains 1 through 5) was isolated and purified as described (34).

Rabbit Polyclonal Antibody to sKDR Receptor (r212). A total of 0.5 mg of purified sKDR protein was injected into the rabbit; after an additional two boosts with 0.2 mg of protein, the animal was bled. The serum revealed a half-maximal titer at a 1:7500 dilution in an enzyme-linked immunosorbent assay for sKDR.

Affinity Purification of r212 Antiserum. Purified KDR protein (900 μg; 110 kD extracellular domain) was coupled to DNBr-activated-Sepharose (Pharmacia Biotech, Freiburg, Germany) with a coupling efficiency of 50%. Then, 4 ml of rabbit antiserum (r212) was applied to this column in 100 mM Tris, 500 mM NaCl, pH 8.0. The column was washed with at least 10 vol of the same buffer. Bound antibodies were eluted with 0.1 M glycin, 150 mM NaCl, pH 2.4, in 500-μl aliquots. The eluted proteins were neutralized at once with 1 M Tris-HCl, pH 8.0. The efficiency of the antibody purification was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis followed by silver staining and Western blot analysis of the purified r212 to KDR. The cross reactivity of the affinity-purified serum with other VEGF receptor proteins was determined by SDS-10% polyacrylamide gel electrophoresis under reducing conditions, followed by Western blotting. Fractionated receptor proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). After the transfer, the Western protocol was then completed as described (31), using affinity-purified r212 at a concentration of 0.55 μg/ml. The bands were visualized using a chromogenic substrate for the alkaline phosphatase-labeled goat anti-rabbit IgG antibody.

Mouse Monoclonal Antibodies to sKDR and Soluble Flt-1 Proteins. Groups of BALB/c mice were immunized with 25 μg of recombinant extracellular sKDR or soluble Flt-1 (sFlt-1) emulsified in Freund's complete adjuvant. Booster injections were given at 3-wk intervals, and fusion between spleen cells of immunized mice and NS-1 mouse myeloma cells was performed 3 d after the third booster injection. Hybridization was induced by exposure of the mixed cells to polyethylene glycol (MW 1500). After fusion, mixed cell cultures were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated horse serum containing 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine

(HAT medium). Cultures were refed with DMEM-HAT medium every 2 to 3 d. After 10 to 14 d, culture supernatants were screened for specific antibodies by enzyme-linked immunosorbent assay and immunoblotting, using baculovirus-expressed KDR extracellular domain or sFlt-1 protein, respectively. Positive wells were isolated, and cells were cloned by limiting dilution. The mouse immunoglobulin isotypes of the clone KDR-1 (IgG-1) and clone 190.11 for anti sFlt-1 (IgG-1) were determined using the Sigma Immunotype TM Kit (ISO-1). The KDR-1 monoclonal antibody did not cross-react with sFlt-1, sFlt-4, or PDGF-Rβ. The Flt-1 monoclonal antibody did not cross-react with sKDR, sFlt-4, or PDGF-Rβ.

Isolation of KDR from Porcine Endothelial Cells Overexpressing the KDR Receptor. Cell lysate from 1×10^7 cells (800 μl) was precleared by incubation with 50 μl of unspecific rabbit serum over 1 h at room temperature (RT) (35). The lysate was then incubated with 50 μl of protein A-Sepharose slurry (saturated with bovine serum albumin) for 30 min at RT and centrifuged in a microcentrifuge for 5 min. The supernatant was then incubated with rabbit anti-KDR serum (diluted 1:20) over 1 h at RT. For the control, the serum for the immunoprecipitation (50 μl) was preincubated with 2.5 μg of purified KDR protein (extracellular domain, 110 kD).

The pellet was washed twice with TSA (10 mM Tris, 10 mM NaCl, pH 8.0), 0.1% Triton, once with TSA, and once with phosphate-buffered saline (PBS). The pellet was dissolved in 30 μl of sample buffer and used for SDS-polyacrylamide gel electrophoresis with 7.5% gels. After blotting, an affinity-purified anti-KDR IgG from rabbit was used, followed by anti-rabbit horseradish peroxidase-conjugated goat IgG (Promega) diluted 1:2500.

Human Kidneys

The adult kidneys ($n = 5$) were received immediately after surgical removal due to renal cell carcinoma. Tumor-free parts were excised, frozen in isopentane, cooled in liquid nitrogen, and stored at −70°C.

The fetal kidneys ($n = 5$) were obtained during autopsies performed shortly after abortion and were handled as described for adult tissue. Only kidneys of fetuses of 17-wk gestation or older were taken for this study; in these, renal glomerular and tubular structures could be well visualized. Gender and age of patients are listed in Table 1. Approval to use sections of these specimens was obtained from the ethics committee of the Medical School of Marburg University.

Immunofluorescence and Immunohistochemistry

Immunohistochemistry was carried out on 5-μm frozen tissue sections fixed in acetone at −10°C for 10 min, using double-label immunofluorescence and double-label immunohistochemistry techniques.

Table 1. Gender and age of patients from whom kidneys were obtained for ^{125}I-VEGF binding studies[a]

Adult		Fetal	
Gender	Age (yr)	Gender	Age (wk)
Female	42	Male	17
Male	46	Male	19
Female	57	Female	20
Female	65	Female	22
Male	63	Female	22

[a] VEGF, vascular endothelial growth factor.

Double-Label Immunofluorescence

Double-label immunofluorescence experiments used the primary rabbit polyclonal anti-KDR antibody (r212) at a 1:10 dilution (containing 35 μg IgG/ml) incubated for 45 min at 37°C, followed by anti-rabbit mouse monoclonal antibody (M 737, DAKO, Hamburg, Germany) for 45 min at 37°C, followed by rhodamine-labeled goat anti-mouse IgG (diluted 1:20, 45 min, 37°C; Biozol, Eching, Germany). Then, after three washes with PBS, fetal calf serum (FCS; 10%) was applied for 20 min at 22°C. Von Willebrand factor antibody (diluted 1:25; DAKO) was incubated for 45 min at 37°C followed by FITC-labeled sheep anti-rabbit IgG (diluted 1:20; Sigma, Munich, Germany). Sections were mounted in aqueous medium.

Double-Label Immunohistochemistry

Tissue sections were incubated with the mouse monoclonal antibody KDR-1 at a concentration of 15 μg IgG/ml or the mouse monoclonal antibody 190.11 to Flt-1 at a concentration of 20 μg IgG/ml. After application of the primary antibody to the tissue sections for 2 h at 22°C, a rabbit anti-mouse antibody (Z 259, DAKO) diluted 1:40 was applied at 22°C for 1 h; alkaline-phosphatase mouse monoclonal antibody (diluted 1:40) was then incubated at 22°C for 1 h. All dilutions were done in PBS, pH 7.6. For staining, sections were exposed to a solution of sodium nitrite (28 mM), new fuchsin (basic fuchsin) (21 mM), naphthol-AS-BI-phosphate (0.5 mM), dimethylformamide (64 mM), and levamisole (5 mM) in 50 mM Tris/HCl buffer, pH 8.4, containing 146 mM NaCl for 15 min.

Sections were then washed in H_2O and PBS, followed by an incubation with FCS (10%). Anti-von Willebrand factor rabbit antibody (DAKO), diluted 1:500, was applied for 18 h at 4°C. Sections were washed three times with PBS at 22°C. A mouse anti-rabbit antibody (M 737, diluted 1:50; DAKO) was applied, followed by a rabbit anti-mouse antibody (Z 259; DAKO) diluted 1:40. Each of these two antibodies was incubated at 22°C for 1 h.

Alkaline-phosphatase mouse monoclonal antibody (diluted 1:40) was then incubated at 22°C for 1 h and developed with Vector Blue R (Camon, Wiesbaden, Germany) to produce a blue color. Sections were mounted in aqueous medium.

Control experiments for the immunhistochemical demonstrations entailed immunohistology with nonimmune mouse IgG or nonimmune rabbit IgG, respectively, and with sKDR or sFlt-1 as competing antigens, without primary antibody, or without alkaline phosphatase antibody. For competition experiments, the respective competitor was added to the section, together with the primary antibody, at a 50-fold higher concentration than that used for the antibody.

VEGF/VPF in Vitro Receptor Binding Assay

Binding experiments were performed on human microvascular endothelial cells (MVEC; Clonetics, San Diego, CA) between passages 6 and 8 and were carried out similar to those described for bovine endothelial cells (36). Briefly, cells were seeded in EGM medium (Clonetics) with 5% FCS (Life Technologies, Paisley, Scotland) at 2×10^4 cells per well in 12-well plates. After 3 to 4 d, the cells were washed using DMEM with 25 mM Hepes, and 1 mg/ml bovine serum albumin, pH 7.4, and incubated for 3 h at 4°C in binding buffer containing 23 pM of ^{125}I-labeled recombinant human $VEGF_{165}$, named ^{125}I-VEGF, with a specific activity of approximately 2000 μCi/ng VEGF (Immundiagnostik, Bensheim, Germany) (31,36). Different amounts of human recombinant $VEGF_{165}$ (33), PLGF-2 (31,33), PDGF-BB, and basic fibroblast growth factor (bFGF) (Sigma) were used for competition experiments (see Figure

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5). Radioactivity present in the lysates was quantified using a gamma counter.

VEGF/VPF in Situ Receptor Binding in Human Kidney

Frozen sections (10 μm; adult and fetal) were cut on a cryostat, thaw-mounted on gelatin-coated glass slides, and placed under vacuum at 4°C overnight. Control sections were stained by hematoxylin-eosin or periodic acid-Schiff to ensure that only tissues free of pathologic changes, determined by light microscopy, were taken. Binding sites for VEGF were labeled by incubation with ^{125}I-VEGF. In short, consecutive sections were preincubated at RT for 30 min in DMEM supplemented with 10% FCS, 25 mM Hepes, 0.5 mM $MgCl_2$, 1 μg/ml leupeptin, and 5 nM phenylmethylsulfonyl fluoride. Sections were then incubated up to 12 h for association experiments and up to 3 h for saturation and competition experiments at RT in the same buffer with the labeled ligand. For saturation experiments with ^{125}I-VEGF, concentrations ranging from 5 to 250 pM were used. Nonspecific binding was determined on alternate sections in the presence of a 700-fold excess of unlabeled VEGF. After incubation, the slides were washed twice for 10 min in PBS, once in 1.5 M NaCl for 15 min to remove unspecific binding to proteoglycans, once in distilled water at RT for 30 s, and then dried under a stream of cold air.

To test the specificity of ^{125}I-VEGF binding in detail, consecutive kidney sections were incubated with 40 pM (adult and fetal kidney) ^{125}I-VEGF and with increasing doses (10^{-12} to 10^{-7} M) of unlabeled VEGF and PLGF-2.

Quantification of ^{125}I-VEGF binding sites was performed by autoradiography with Hyperfilm-^{3}H (Amersham-Buchler, Braunschweig, Germany). The sections were exposed for 2 d, together with [^{125}I]-standards (microscales, Amersham-Buchler). The films were densitometrically analyzed using a computerized image analysis system (MCID Imaging, St. Catharines, Ontario, Canada). Grey values of the [^{125}I]-microscales were used to determine the amount of ^{125}I-VEGF bound to tissue sections, which was expressed in fmol bound per mg tissue equivalent. The validity of this method for quantification of peptide binding sites in tissue sections has been established (37).

The equilibrium dissociation constant (K_d) and the maximal number of binding sites (B_{max}) were derived from saturation experiments. Data were generated with curve-fitting programs, using Scatchard analysis and nonparametric regression analysis (GraphPad Prism, Graph Pad Software, San Diego, CA).

To define the anatomical relationships between ^{125}I-VEGF binding sites and renal structures more precisely, we used the coverslip technique. After incubation of renal sections with 50 pM ^{125}I-VEGF, the dry sections were covered with Kodak NTB 2 emulsion (Kodak, Rochester, NY). Slides were stored at 4°C for 10 to 14 d, developed in Kodak D19, fixed in Kodak Readymatic, and counterstained with eosin.

Statistical Analyses

Data are given as means ± SD. The unpaired *t* test was used to determine statistical significance with a two-tailed *P* value of <0.01 regarded as a significant difference between groups (Instat, GraphPad Software).

Results

Reactivity of the Rabbit Polyclonal Antibody to sKDR

In isolated cell lysate from porcine endothelial cells overexpressing the KDR receptor, the polyclonal antibody precipitated KDR proteins in the range of 190 to 220 kD; this is in good agreement with unglycosylated and glycosylated forms of the human KDR protein (35). Preincubation of the antibody with purified extracellular domain of KDR (110 kD) led to a disappearance of the specific immunobands (Figure 1A).

This polyclonal antibody also detected, at a moderate level, the FLT-4 protein, a receptor that is expressed in lymphatic endothelial cells. Also, a weak cross reactivity with the second VEGF receptor (FLT-1) was noticed. Other receptor proteins that are not related to the VEGF receptor but belong to the same class III receptor tyrosine kinases such as PDGF-Rβ were not recognized by the antibody (Figure 1B).

Localization of VEGF-Receptor Proteins

Fetal Kidney. Double-label immunofluorescence for VEGF-R proteins with the anti-KDR antibody r212, which was slightly cross-reactive with VEGF-R1 (Flt-1), depicted VEGF-R protein in comma-shaped and S-structures as soon as endothelial cells positive for von Willebrand factor appeared (Figure 2, a through d). VEGF-R could not be demonstrated in cells that were negative for von Willebrand factor. In more developed stages of glomerular convolutes, positive label for VEGF-R, Flt-1 (Figure 3a), and KDR (Figure 4a) colocalized with von Willebrand factor, but with the latter antigen expressed often more strongly than VEGF-R (Figure 2, c and d, and Figures 3a and 4a). Peritubular capillaries also had a coexpression of VEGF-R and von Willebrand factor. Comparable results for fetal glomeruli were obtained with the monoclonal antibodies specific for KDR or Flt-1 protein (Figures 3a and 4a).

Adult Kidney. In human adult kidney, glomerular capillaries (Figures 3b and 4c) and endothelial cells of arteries (Figure 3c) were positive for VEGF-R. As shown by the monoclonal antibodies, only endothelial cells were stained, also in peritubular capillaries (Figures 3d and 4b). Mesangial cells and vascular smooth muscle cells did not exhibit staining for VEGF receptors.

Binding Characteristics of VEGF Receptors

Human MVEC in Culture. To analyze whether iodinated $VEGF_{165}$ binds specifically to the VEGF/VPF cell surface receptors, the radioactive ligand was incubated with human MVEC. The binding could be competed in the excess of unlabeled $VEGF_{165}$ or PLGF-2. Fifty percent inhibition of the specific binding was achieved with 58 pM $VEGF_{165}$ and 8 nM PLGF-2. PDGF and bFGF did not compete for VEGF binding (Figure 5).

Human Kidney. Photomicrographs of whole fetal and adult kidneys demonstrated a strong binding of ^{125}I-VEGF to glomeruli, peritubular capillaries, and vascular bundles of the medulla. Autoradiographic label of preglomerular vessels was less pronounced (Figure 6, A and C). Unlabeled VEGF completely abolished binding in adult and fetal kidneys (nonspecific binding $< 1\%$; Figure 6, B and D).

By light microscopy, a strong signal in endothelium could be discerned in postglomerular peritubular capillaries and in veins in adult kidney; the endothelial label of arteries and afferent arterioles was distinct but rather weak. Glomerular binding of ^{125}I-VEGF was very high; because of the intensity of the

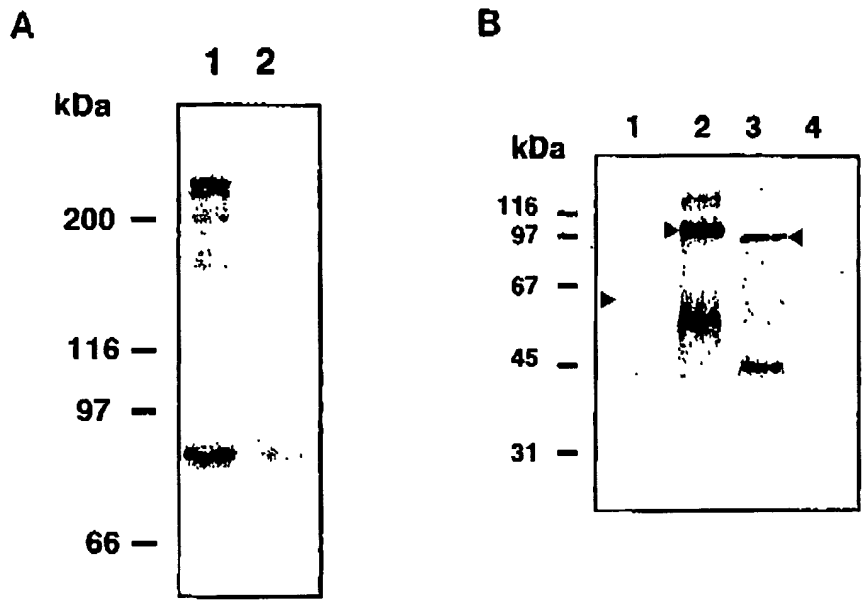

Figure 1. (A) Western blot of cell lysate after immunoprecipitation from PAE/ kinase insert domain receptor (KDR) cells probed with affinity-purified rabbit anti-KDR r212 (lane 1); preincubated before immunoprecipitation with 25 μg of KDR protein (lane 2). Molecular weight standards (shown at left): $\times 10^3$. Bands approximately 180 to 220 kD (lane 1) are in agreement with those predicted for KDR glycosylated and unglycosylated proteins. (B) Other purified extracellular receptor tyrosine kinases were analyzed for reactivity with the polyclonal antibody r212 by electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting. The molecular mass of marker proteins is shown at left. Lane 1, 50 ng of soluble FLT-1 (sFLT-1) (vascular endothelial growth factor [VEGF-R1]); lane 2, 50 ng of soluble KDR-1 (sKDR-1) (VEGF-R2); lane 3, 200 ng of semipure sFLT-4 (VEGF-R3); lane 4, 50 ng of PDGF-Rβ. Pronounced reactivity was seen with sKDR-1 (lane 2, arrowhead) and its low molecular weight degradation product. Cross reactivity could be detected with sFlt-4 (lane 3, arrowhead) and with sFlt-1 (lane 1, arrowhead).

radiographic signals, it could not be definitely assigned solely to endothelial cells (Figure 6, E and F). In fetal human kidney, ^{125}I-VEGF binding sites were present in pre- and postglomerular vessels and in developing glomeruli, as soon as capillary structures were formed. Avascular structures, tubules, and mesenchyme did not bind VEGF (Figure 6, G and H).

The kinetics (K_d, B_{max}) of ^{125}I-VEGF binding are depicted in Figure 7, A through H, and Table 2 for glomeruli, cortex, and medulla of adult and fetal kidneys. Association experiments with ^{125}I-VEGF revealed complete saturation for all renal structures of adult and fetal kidneys after 3 to 4 h (Figure 7, A and B). Glomeruli exhibited the highest binding capacity, whereas cortical and medullary vessels had a higher binding affinity. Adult kidneys consistently showed a larger B_{max} than fetal kidneys; however, there were no differences in the affinity between fetal and adult renal parenchyma (Figure 7, C and D, Table 2).

Displacement of ^{125}I-VEGF by unlabeled VEGF was complete, whereas PLGF-2 demonstrated competition for binding sites by more than 60% in all renal structures examined (Figure 7, E through H). Because experiments in cultured microvascular endothelial cells did not indicate a displacement of VEGF binding sites by PDGF or bFGF, these studies were not repeated in fetal and adult kidney sections.

Discussion

In contrast to other angiogenic growth factors such as bFGF, VEGF/VPF seems to be a mitogen with a high specificity for endothelial cells (5,38,39). VEGF/VPF acts on two transmembrane receptors and plays an important role in blood vessel formation during development. It has also been implicated in angiogenesis during tumor growth, wound healing, diabetic retinopathy, atherosclerosis, and rheumatoid arthritis (40–43). VEGF and its receptors are highly expressed during organogenesis of most fetal organs, including the kidney (4,12,44). In addition, the adult kidney constitutively expresses VEGF mRNA and protein and VEGF receptor mRNA (11,12). In a recent study, it was demonstrated that VEGF mRNA and protein are overexpressed (*e.g.*, proximal tubules) during hypoxia of the kidney (45). In view of these findings and of the known multiple actions of VEGF, including strong enhancement of microvascular permeability, this growth factor may be involved in regulation of normal kidney physiology, as well as in renal pathophysiologic conditions.

To better understand the possible role of VEGF in the kidney, we have analyzed for the first time ^{125}I-VEGF binding sites and expression of VEGF receptor protein by immunohistology in different developmental stages of the human kidney. Specific binding sites were documented on preglomerular ves-

Figure 2. (a through d
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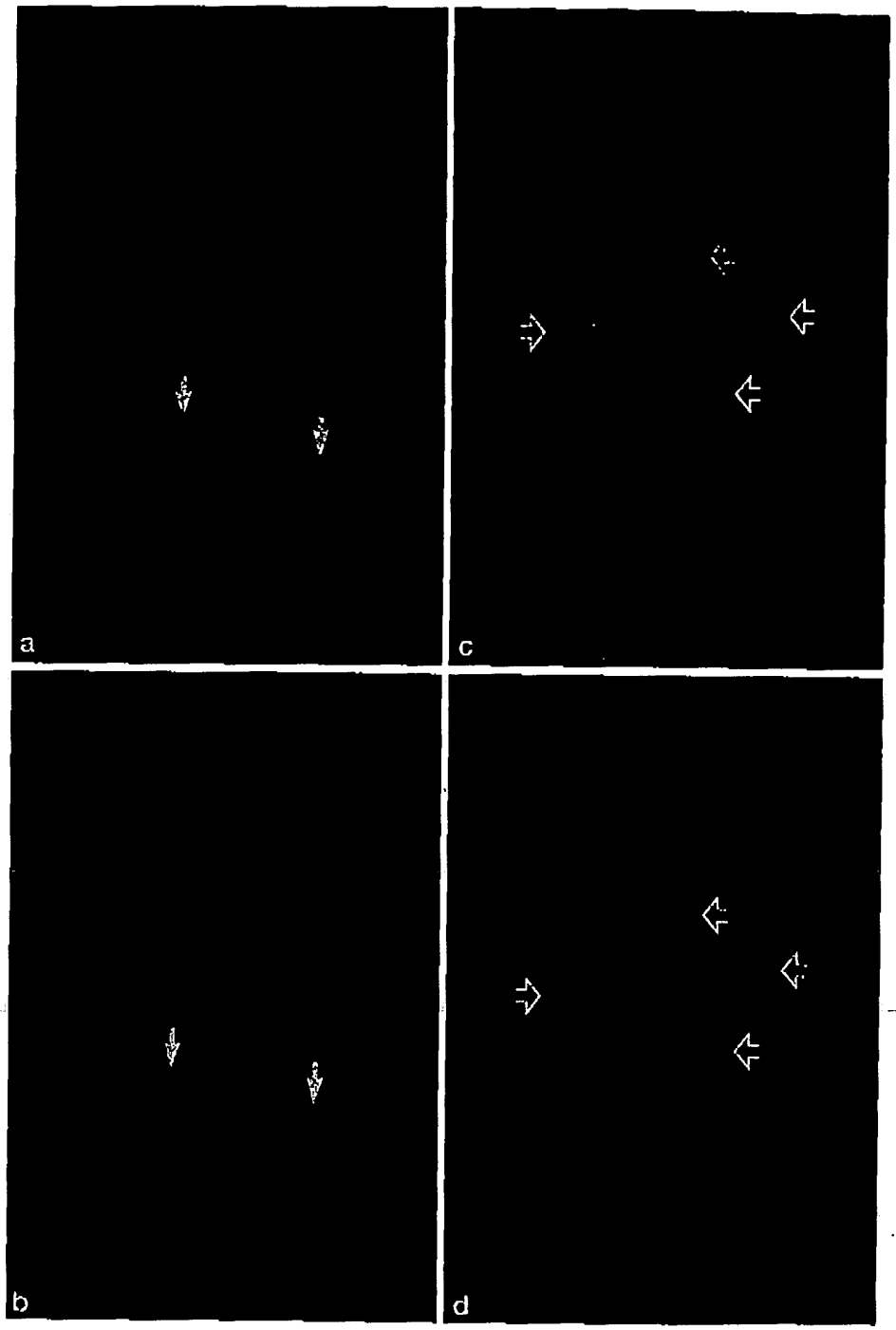

Figure 2. (a through d) Double-label immunofluorescence for VEGF-receptor (R) proteins with an affinity-purified polyclonal rabbit antibody 7-12 (rhodamine, a and c) and the endothelial antigen von Willebrand factor (FITC, b and d) in human developing glomerular structures. (a through d) Fetal kidney (male, 17 wk). Comma-shaped structure in subcapsular area with slit-like coexpression of VEGF-R proteins (a) and von Willebrand factor (b) (arrows). (c and d) Advanced stage of development of glomerular convolute with colocalization of VEGF-R proteins (c) and von Willebrand factor (d) in capillary structures (arrowheads).

) cells probed with (lane 2). Molecular predicted for KDR reactivity with the by Western blotting. ıelial growth factor); lane 4, 50 ng of tion product. Cross-

ors such as bFGF. igh specificity for ɔn two transmem- e in blood vessel een implicated in healing, diabetic arthritis (40–43). :d during organo- dney (4,12,44). In expresses VEGF NA (11,12). In a EGF mRNA and ıules) during hyp- ıdings and of the ; strong enhance- vth factor may be iology, as well as

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ls, glomeruli, peritubular capillaries, and medullary vascular ys. Considering the exclusive demonstration of VEGF receptor proteins on endothelial cells by immunohistology, binding sites seem to be restricted to endothelial cells. The detailed studies by Robert *et al.* documented cells expressing flk-1, the murine homologue to KDR, in the nephrogenic mesenchyme in

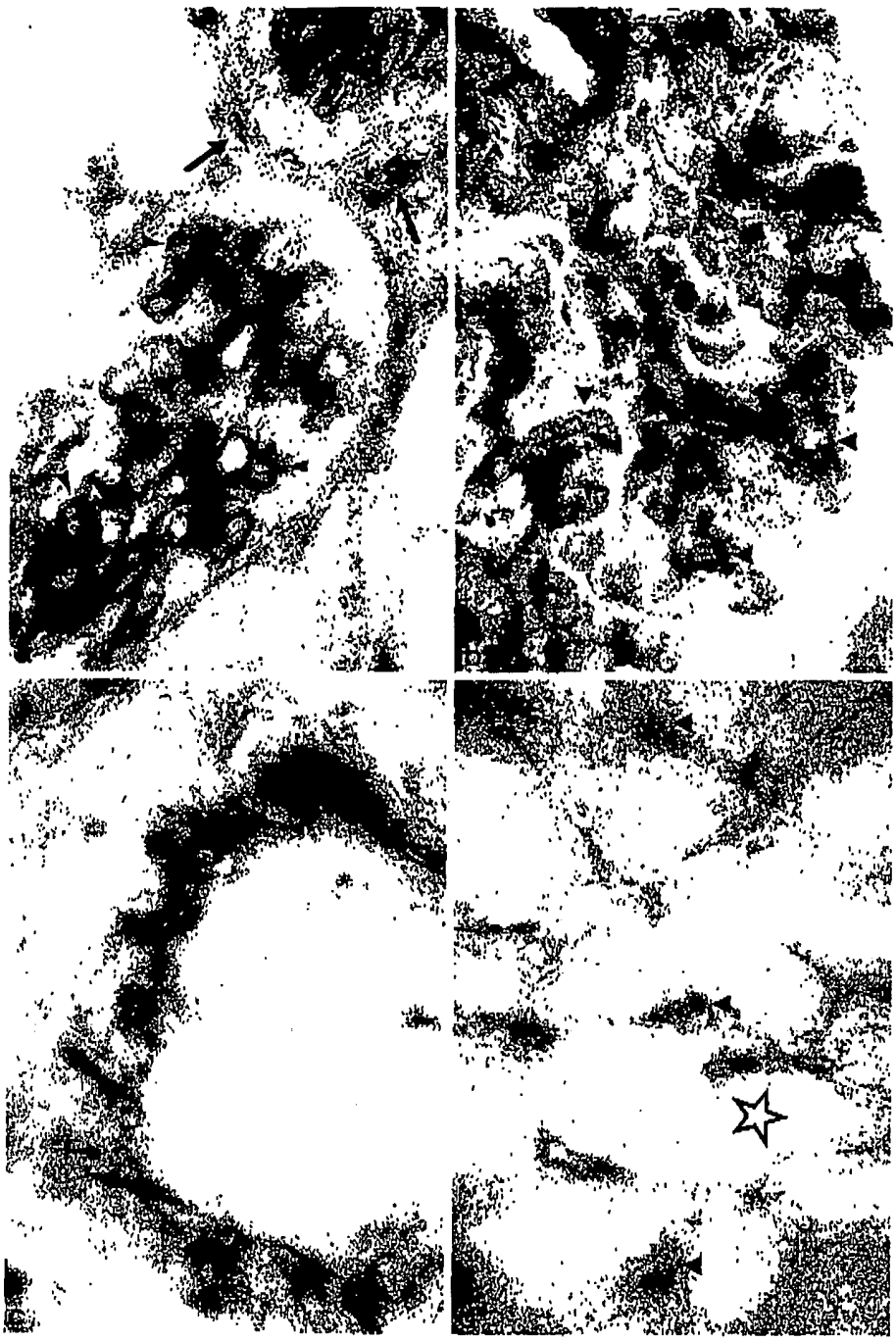

Figure 3. (a through d) Double-label immunohistology for VEGF-R protein (Flt-1) with monoclonal mouse antibody 190.11 and for the endothelial antigen von Willebrand factor. (a) Fetal kidney (female, 20 wk). Glomerulus with a red positive label for Flt-1 protein in capillaries (arrowheads) that are also positive for von Willebrand factor (blue). Periglomerular capillaries also showed colocalization of Flt-1 and von Willebrand factor (arrows). (b through d) Adult kidney (male, 46 yr). (b) Part of glomerulus with capillaries that are positive both for Flt-1 (red) and von Willebrand factor (blue) (arrowheads). (c) Part of interlobular artery with colocalization of Flt-1 (red) and von Willebrand factor (blue) to endothelium; media is negative. (d) Peritubular capillaries are positive for Flt-1 (red) and von Willebrand factor (blue) (arrowheads). Tubular epithelia are negative (star). For technical details, see Materials and Methods.

the mouse (46). In the current study, angioblast-like KDR-positive cells could not be seen scattered in the subcapsular zone of fetal kidney, not even by taking a polyclonal antibody strongly reactive to KDR, Flt-4, and Flt-1. It cannot be excluded that this phenomenon of KDR expressing angioblasts may be detectable in very early stages of human nephrogen-

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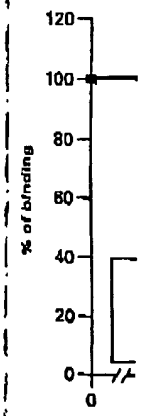

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Figure 4. (a through c) Double-label immunohistology for VEGF-R (KDR) protein with a monospecific mouse monoclonal antibody KDR-1 and the endothelial antigen von Willebrand factor. (a) Fetal kidney (male, 17 wk). Glomerulus with a red positive label for KDR protein in capillaries (arrowheads) that are also positive for von Willebrand factor (blue). (b and c) Adult kidney (female, 42 yr). (b) Peritubular capillaries in cortex with blue label for von Willebrand factor are also positive for KDR (red signal) (arrowheads). Tubular epithelia did not stain (stars). (c) Part of glomerulus with capillaries that are showing both labels (red and blue) for KDR and von Willebrand factor, respectively (arrowheads). For technical details, see Materials and Methods.

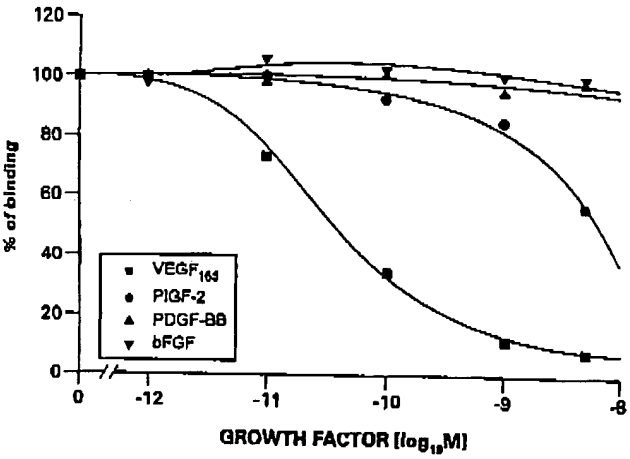

Figure 5. Competition of ^{125}I-VEGF$_{165}$ (23 pM) binding to human microvascular endothelial cells by unlabeled VEGF$_{165}$ and unlabeled human recombinant placenta growth factor (PLGF)-2. Platelet-derived growth factor and basic fibroblast growth factor did not influence ^{125}I-VEGF$_{165}$ binding.

esis. Another possibility might be a species difference as described for receptors of vasoactive peptides (28,29).

Quantitative autoradiography, using nonparametric regression analysis, revealed that ^{125}I-VEGF binding sites were highly specific, saturable, and of high affinity. These findings were comparable to the specificity and potency of VEGF binding observed during *in vitro* studies in cultured endothelium. The binding was reversible and could be displaced by unlabeled VEGF. Linear regression of the Scatchard plots revealed a parabolic shape for the first concentrations, suggesting a mixed receptor pattern with negative receptor cooperation (Figure 7). However, using a nonparametric regression analysis for both a one-binding site and a two-binding site model, only a one-binding site model showed high correlation coefficients for all concentrations. Interestingly, very similar binding parameters could be calculated using linear regression of Scatchard plots, omitting the first three concentrations. This suggests that in the kidney, Flt-1 and KDR have similar binding characteristics for VEGF, although a slight difference in binding affinity or a negative receptor cooperation in the lower concentration range cannot be definitely excluded. The closely related growth factor PLGF, known to bind to the Flt-1 receptor but not to KDR, displaced the ^{125}I-VEGF binding by approximately 60%, indicating that the Flt-1 receptors are slightly more prevalent than the KDR receptors in the different developmental stages of the human kidney.

VEGF binding sites were strongly expressed in adult human kidneys in which vasculo- and angiogenesis was not occurring. This was confirmed by a positive immunohistology for VEGF receptor protein in renal endothelial cells not only in fetal, but also in adult kidneys. Mesangial cells or vascular sm oth muscle cells could not be shown to hav VEGF receptor protein. This observation differs from that of the absence of

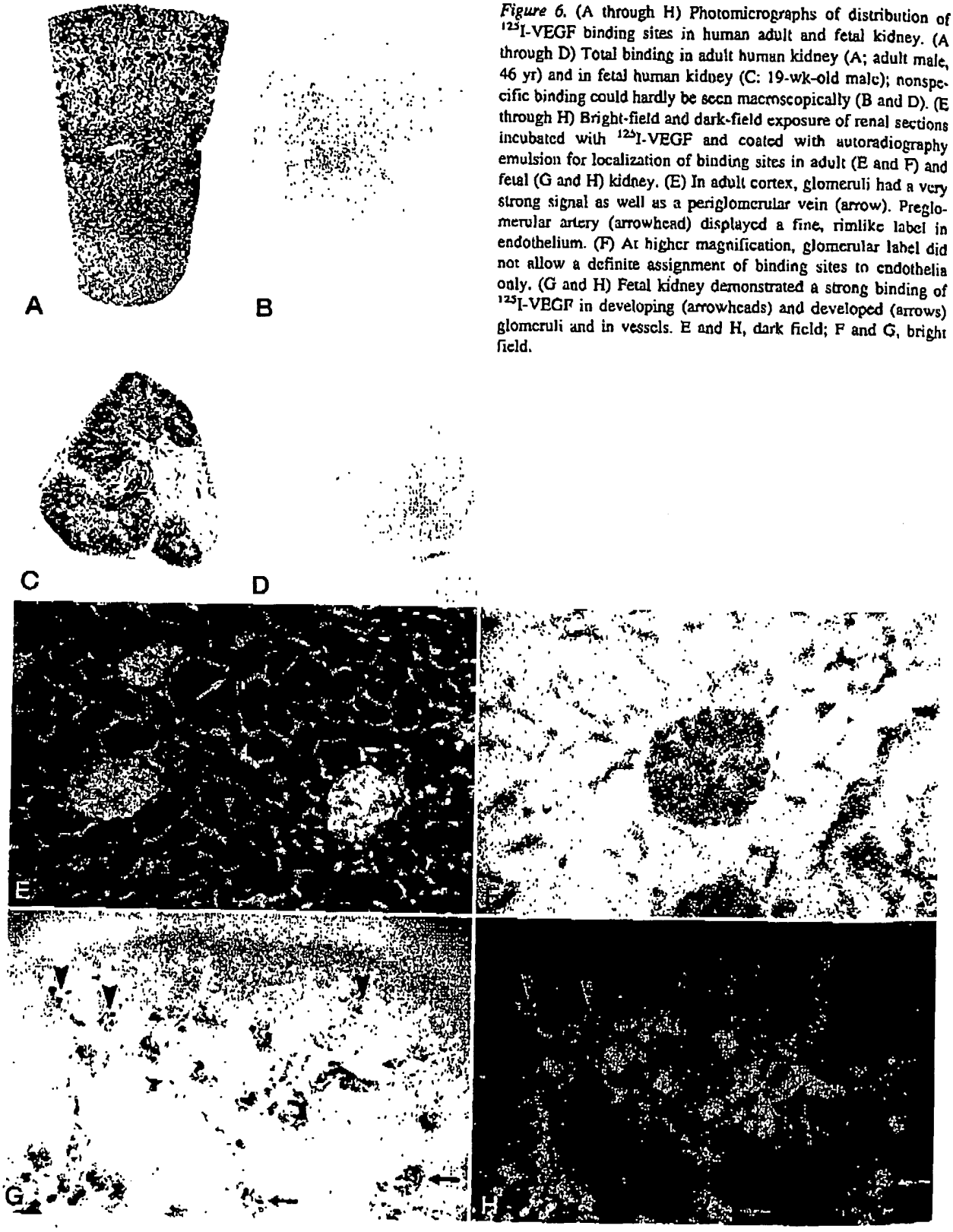

Figure 6. (A through H) Photomicrographs of distribution of ^{125}I-VEGF binding sites in human adult and fetal kidney. (A through D) Total binding in adult human kidney (A; adult male, 46 yr) and in fetal human kidney (C: 19-wk-old male); nonspecific binding could hardly be seen macroscopically (B and D). (E through H) Bright-field and dark-field exposure of renal sections incubated with ^{125}I-VEGF and coated with autoradiography emulsion for localization of binding sites in adult (E and F) and fetal (G and H) kidney. (E) In adult cortex, glomeruli had a very strong signal as well as a periglomerular vein (arrow). Preglomerular artery (arrowhead) displayed a fine, rimlike label in endothelium. (F) At higher magnification, glomerular label did not allow a definite assignment of binding sites to endothelia only. (G and H) Fetal kidney demonstrated a strong binding of ^{125}I-VEGF in developing (arrowheads) and developed (arrows) glomeruli and in vessels. E and H, dark field; F and G, bright field.

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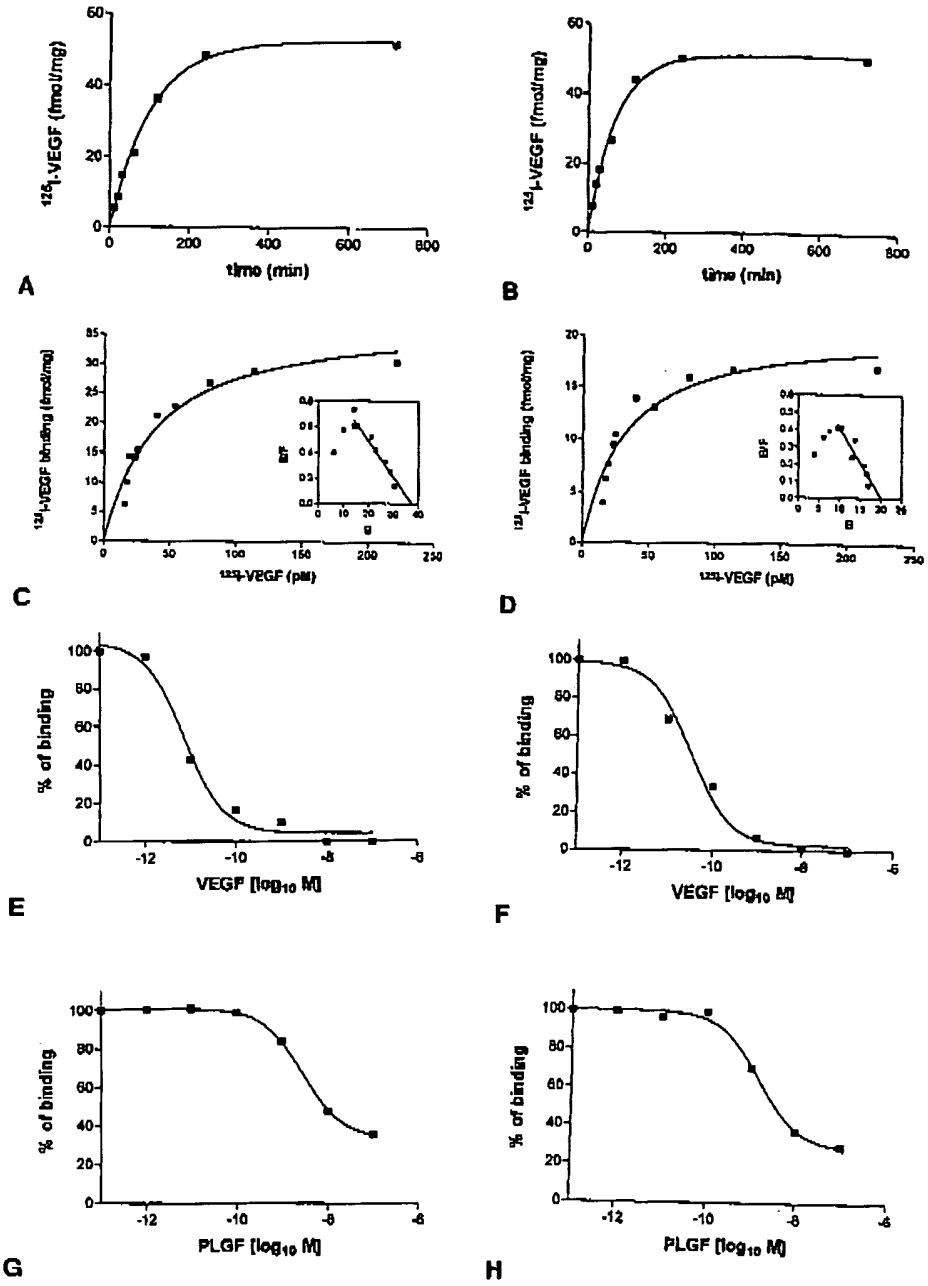

Figure 7. (A through H) Association, saturation, and inhibition curves of specific ^{125}I-VEGF binding to glomeruli of adult and fetal human kidney as calculated by nonparametric regression. (A and B) Association of ^{125}I-VEGF to adult kidney glomeruli revealed saturation after 4 h (A). Similar results were obtained for fetal kidney (B). (C and D) Saturation curves of specific binding of ^{125}I-VEGF to adult (C) and fetal (D) glomeruli. Scatchard plots of data are shown in insets in C and D. (E through H) Competition with unlabeled VEGF and with PLGF-2 in adult (E and G) and fetal (F and H) glomeruli showing complete competition by VEGF and more than 60% inhibition by PLGF-2. Data shown are from a representative experiment. Similar results were obtained for five adult and five fetal kidneys.

VEGF/VPF receptors in other tissues in which the receptors are downregulated in quiescent endothelial cells but highly upregulated in neoplastic tissue or tissue undergoing angiogenesis (*e.g.*, female reproductive system) (47). The constitutive expression of VEGF receptors on endothelia of adult kidney hints at nonangiogenic renal actions of VEGF. It has been found that VEGF can be vasodilatory in the isolated perfused rat kidney (48) and chemotactic (49).

VEGF/VPF is known to be a strong enhancer of microvascular permeability, being 50,000 times more potent than histamine (38,40). VEGF/VPF may induce microvascular hyperpermeability by activating vesicular–vacuolar organelles in the

Table 2. Affinity (K_d) and maximal capacity of binding (B_{max}) for ^{125}I-VEGF$_{165}$ in different structures of fetal and adult human kidney[a]

Group	K_d (pmol)	B_{max} (fmol/mg)	IC_{50} VEGF (pmol)	IC_{50} PLGF (nmol)	Half Time Assoc. (min)
Adult					
glomeruli	38.64 ± 11.20	40.85 ± 5.36[b]	6.40 ± 1.70[b]	7.42 ± 3.51	56.28 ± 9.59
cortical tubulointerstitium	19.36 ± 2.61	6.38 ± 1.26	4.55 ± 0.99	8.98 ± 4.97	29.19 ± 4.75
medullary tubulointerstitium	19.13 ± 1.72	4.79 ± 1.18	5.90 ± 1.79	10.77 ± 5.58	36.61 ± 6.18
Fetal					
glomeruli	36.26 ± 7.10	24.02 ± 3.81	22.76 ± 8.58	7.90 ± 4.84	41.53 ± 4.64
tubulointerstitium	11.60 ± 7.00	2.92 ± 0.51	27.73 ± 2.87	5.29 ± 2.87	30.65 ± 6.21

[a] IC_{50}, 50% inhibition of specific binding; PLGF, placenta growth factor; assoc., association.
[b] $P < 0.01$ to corresponding fetal value.

cytoplasm of venular endothelia, by stimulating formation of interendothelial gaps, or by forming endothelial fenestrations (6,40,50). It has been hypothesized that VEGF is involved in the maintenance of fenestrae in renal glomerular and peritubular endothelial cells because of its persistent expression in adjacent epithelial cells, (*e.g.*, podocytes and collecting duct cells) (12). Our finding of specific, high-affinity binding sites for VEGF on glomerular and tubulointerstitial capillary fenestrated endothelium strengthens the hypothesis that VEGF produced in podocytes and collecting duct cells may act in a paracrine manner to exert its effect on kidney microvessels. Competition experiments demonstrated that the majority of renal VEGF receptors are Flt-1 receptor type. Recently, it has been reported that Flt-1-deficient mice formed abnormal embryonic vasculature and die *in utero* at approximately 8 d p.c. (22). In flk-deficient mice, mature endothelial cells or organized blood vessels could not be observed in the embryo at any stage of development (21). The authors suggested that Flt-1 may be important for the organization of embryonic vasculature regulating endothelial cell–cell and/or cell–matrix interactions, whereas flk (analogous to KDR) may be important for vasculogenesis, endothelial cell growth, and differentiation.

A recent electron microscopic study, using topical application and intradermal injection of recombinant human VEGF, has shown that VEGF may increase microvascular leakage by opening endothelial intercellular junctions and inducing fenestrae in capillary endothelia, which are normally not fenestrated (50).

In the kidney, the regulation of the number of fenestrae and endothelial intercellular junctions, therefore, might involve the VEGF receptor system. However, this possibility has not yet been confirmed by *in vivo* experiments in the kidney. The immunohistologic finding of a more pronounced staining for VEGF receptors in glomeruli and postglomerular capillaries compared with preglomerular vessels may be taken as an additional indication of such an action of VEGF.

In the adult kidney, angiogenesis is rarely observed, even during pathologic processes; hence, a different pattern of VEGF receptor expression between fetal and adult kidney could have been expected. However, during all of the developmental stages examined, the receptor protein expression pattern did not vary. In addition, no differences in the binding affinity to VEGF between fetal and adult kidney could be found. In contrast, maximal binding capacity seemed to increase in the adult kidney. The significantly higher B_{max} of adult glomeruli than of fetal glomeruli is probably due to the higher density of capillary segments, *e.g.*, endothelial cells per area in adult than in developing glomeruli. Nevertheless, it is tempting to speculate that VEGF might act differently in kidney development and adult kidney by using different dominant signaling mechanisms. Different signaling pathways for Flt-1 and KDR have been demonstrated recently *in vitro*, using porcine aortic endothelial cells and human umbilical vein endothelial cells (35).

In summary, VEGF receptor proteins and specific ^{125}I-VEGF binding sites could be demonstrated in different developmental stages of the human kidney. Receptor proteins and binding could be localized to endothelial cells of preglomerular vessels, glomeruli, medullary vascular rays, and the tubulointerstitial capillary network.

Competition experiments using PLGF indicated that Flt-1 was the most abundant VEGF receptor in the kidney. These findings may aid in the interpretation of functional studies. Our data also support the view that VEGF exerts additional actions, such as an action on endothelial integrity in the kidney, that differ from its mitogenic effects on endothelium.

Acknowledgments

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References

1. Schwartz MM, Venkatachalam MA: Ultrastructural differences between rat inner medullary descending and ascending vasa recta. *Lab Invest* 35: 161–170. 1976

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1. Brenner BM, Deen WM, Robertson CR: Glomerular filtration. In: *The Kidney*, edited by Brenner BM, Rector FC Jr, Philadelphia, W. B. Saunders, 1976, pp 251–271
2. Breier G, Albrecht U, Sterrer S, Risau W: Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development* 114: 521–532, 1992
3. Peters KG, De Vries C, Williams LT: Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. *Proc Natl Acad Sci USA* 90: 8915–8919, 1993
4. Ferrara N, Henzel WJ: Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 161: 851–858, 1989
5. Senger DR: Molecular framework for angiogenesis: A complex web of interactions between extravasated plasma proteins and endothelial cell proteins induced by angiogenic cytokines. *Am J Pathol* 149: 1–7, 1996
6. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A: Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380: 435–439, 1996
7. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW: Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380: 439–442, 1996
8. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW: The vascular endothelial growth factor family: Identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol* 5: 1806–1814, 1991
9. Park JE, Keller GA, Ferrara N: The vascular endothelial growth factor (VEGF) isoforms: Differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol Biol Cell* 4: 1317–1326, 1993
10. Brown LF, Berse B, Tognazzi K, Manseau EJ, Van de Water L, Senger DP, Dvorak HF, Rosen S: Vascular permeability factor mRNA and protein expression in human kidney. *Kidney Int* 42: 1457–1461, 1992
11. Simon M, Grône HJ, Jöhren O, Kullmer J, Plate KH, Fuchs E, Risau W: Expression of vascular endothelial growth factor and its receptors in human renal ontogenesis and in adult kidney. *Am J Physiol* 268: F240–F250, 1995
12. Kloth S, Ebenbeck C, Kubitza M, Schmidbauer A, Röckl W, Minuth WW: Stimulation of renal microvascular development under organotypic culture conditions. *FASEB J* 9: 963–967, 1995
13. Kitamoto Y, Tokunaga H, Tomita K: Vascular endothelial growth factor is an essential molecule for mouse kidney development: Glomerulogenesis and nephrogenesis. *J Clin Invest* 99: 2351–2357, 1997
14. Clauss M, Gerlach M, Gerlach H, Brett J, Wang F, Familetti PC, Pan YCE, Olander JV, Connolly DT, Stern D: Vascular permeability factor: A tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity and promotes migration. *J Exp Med* 172: 1535–1545, 1990
15. Unemori EN, Ferrara N, Bauer EA, Amento EP: Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. *J Cell Physiol* 153: 557–562, 1992
16. Pepper MS, Ferrara N, Orci L, Montesano R: Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. *Biochem Biophys Res Commun* 181: 902–906, 1991
17. Mandriota SJ, Seghezzi G, Vassalli JD, Ferrara N, Wasi S, Mazzieri R, Mignatti P, Pepper MS: Vascular endothelial growth factor increases urokinase receptor expression in vascular endothelial cells. *J Biol Chem* 270: 9709–9716, 1995
18. De Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT: The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 255: 989–991, 1992
19. Millauer B, Wizigmann-Voos S, Schnürch H, Martinez R, Moller NPH, Risau W, Ullrich A: High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 72: 835–846, 1993
20. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitmann ML, Schuh AC: Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376: 62–66, 1995
21. Fong GH, Rossant J, Gertsenstein M, Breitman ML: Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 376: 66–70, 1995
22. Oberg C, Waltenberger J, Claesson WL, Welsh M: Expression of protein tyrosine kinases in islet cells: Possible role of the Flk-1 receptor for beta cell maturation from duct cells. *Growth Factors* 10: 115–126, 1994
23. Takahashi T, Shirasawa T, Miyake K, Yahagi I, Maruyama N, Kasahara N, Kawamira T, Matsumura O, Mitarai T, Sakai O: Protein tyrosine kinases expressed in glomeruli and cultured glomerular cells: Flt-1 and VEGF expression in renal mesangial cells. *Biochem Biophys Res Commun* 209: 218–226, 1995
24. Brown LF, Detmar M, Tognazi K, Abu-Jawdek G, Iruela-Arispe ML: Uterine smooth muscle cells express functional receptors (flt-1 and KDR) for vascular permeability factor/vascular endothelial growth factor. *Lab Invest* 76: 245–255, 1997
25. Cohen T, Gitay-Goren H, Sharon R, Shibuya M, Halaban R, Levi B-Z, Neufeld G: $VEGF_{121}$, a vascular endothelial growth factor (VEGF) isoform lacking heparin binding ability, requires cell-surface heparan sulfates for efficient binding to the VEGF receptors of human melanoma cells. *J Biol Chem* 270: 11322–11326, 1995
26. Jakeman LB, Winer J, Bennett GL, Altar CA, Ferrara N: Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. *J Clin Invest* 89: 244–253, 1992
27. Fuchs E, Grönc HJ, Simon M, Laue A: Distribution of [^{125}I] endothelin binding sites in mammalian kidneys. *Comp Biochem Physiol* 101A: 775–778, 1992
28. Gröne HJ, Simon M, Fuchs E: Autoradiographic characterization of angiotensin receptor subtypes in fetal and adult human kidney. *Am J Physiol* 262: F326–F331, 1992
29. Maglione D, Guerriero V, Viglietto G, Ferraro MG, Aprelikova O, Alitalo K, Del Vecchio S, Lei KJ, Chou JY, Persico MG: Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PLGF), are transcribed from a single gene of chromosome 14. *Oncogene* 4: 925–931, 1993
30. Hauser S, Weich HA: A heparin-binding form of placenta growth factor (PLGF-2) is expressed in human umbilical vein endothelial cells and in placenta. *Growth Factors* 9: 259–268, 1993
31. Terman B, Khandke L, Dougher-Vermazan M, Maglione D, Lassam NJ, Gospodarowicz D, Persico MG, Böhlen P, Eisinger M: VEGF receptor subtypes KDR and Flt-1 show different sensitivities to heparin and placenta growth factor. *Growth Factors* 11: 187–195, 1994
32. Clauss M, Weich H, Breier G, Knies U, Röckl W, Waltenberger J, Risau W: The vascular endothelial growth factor receptor flt-1 mediates biological activities: Implications for a functional role

of placenta growth factor in monocyte activation and chemotaxis. *J Biol Chem* 271: 17629–17634, 1996

34. Barleon B, Totzke F, Herzog C, Blanke S, Kremmer E, Siemeister G, Marme D, Martiny-Baron G: Mapping of the sites for ligand binding and receptor dimerization at the extracellular domain of the vascular endothelial growth factor receptor Flt-1. *J Biol Chem* 272: 10382–10388, 1997
35. Waltenberger J, Claesson WL, Siegbahn A, Shibuya M, Heldin C-H: Different signal transduction properties of KDR and flt-1, two receptors for vascular endothelial growth factor. *J Biol Chem* 269: 26988–26995, 1994
36. Fiebich BL, Jäger B, Schöllmann C, Weindel K, Wilting J, Kochs G, Marme D, Hug H, Weich HA: Synthesis and assembly of functionally active human vascular endothelial growth factor homodimers in insect cells. *Eur J Biochem* 211: 19–26, 1993
37. Davenport AP, Hall MD: Comparison between brain paste and polymer [^{125}I] standards for quantitative receptor autoradiography. *J Neurosci Methods* 5: 75–82, 1988
38. Ferrara N, Houck K, Jakeman L, Leung DW: Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr Rev* 13: 18–32, 1992
39. Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J: Tumor vascular permeability factor stimulates endothelial growth and angiogenesis. *J Clin Invest* 84: 1470–1478, 1989
40. Dvorak HF, Brown LF, Detmar M, Dvorak AM: Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 146: 1029–1039, 1995
41. Berkman RA, Merrill MJ, Reinhold WC, Monacci WT, Saxea A, Clark WC, Robertson JT, Ali IU, Oldfield EH: Expression of the vascular permeability factor/vascular endothelial growth factor gene in central nervous system neoplasms. *J Clin Invest* 91: 153–159, 1993
42. Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE: Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 331: 1480–1487, 1994
43. Koch AE, Harlow LA, Haines GK, Amento EP, Unemori EN, Wong WL, Pope RM, Ferrara N: Vascular endothelial growth factor: A cytokine modulating endothelial function in rheumatoid arthritis. *J Immunol* 152: 4149–4156, 1994
44. Kaipainen A, Korhonen J, Pajusola K, Aprelikova O, Persico MG, Terman BI, Alitalo K: The related flt 4, flt 1 and KDR receptor tyrosine kinases show distinct expression patterns in human fetal endothelial cells. *J Exp Med* 178: 2077–2088, 1993
45. Gröne HJ, Simon M, Gröne EF: Expression of vascular endothelial growth factor in renal vascular disease and allografts. *J Pathol* 177: 259–267, 1995
46. Robert B, St. John PL, Hyink DP, Abrahamson DR: Evidence that embryonic kidney cells expressing flk-1 are intrinsic, vasculogenic angioblasts. *Am J Physiol* 271: F744–F753, 1996
47. Cullinan-Bove K, Koos RD: Vascular endothelial growth factor/vascular permeability factor expression in the rat uterus: Rapid stimulation by estrogen correlates with estrogen-induced increases in uterine capillary permeability and growth. *Endocrinology* 133: 829–837, 1993
48. Klanke B, Simon M, Röckl W, Weich HA, Stolte H, Gröne HJ: Effects of vascular endothelial growth factor (VEGF) vascular permeability factor (VPF) on hemodynamics and permselectivity of the isolated perfused rat kidney. *Nephrol Dial Transplant* 13: 875–885, 1998
49. Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D: Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood* 3: 3336–3343, 1997
50. Roberts WG, Palade GE: Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. *J Cell Sci* 108: 2369–2379, 1995

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